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Overexpression, Purification, and Characterization of the Herpes Simplex Virus-1 DNA Polymerase-UL42 Protein Complex

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The herpes simplex virus type-1 (HSV-1)-encoded DNA polymerase consists of two subunits, the products of the UL30 and UL42 genes. UL30 and UL42 were coexpressed in Sf9 cells infected with recombinant baculoviruses carrying the two genes. The UL30 and UL42 gene products remained tightly associated throughout the purification, which led to a near homogeneous heterodimer composed of the DNA polymerase and UL42 protein. The DNA polymerase-UL42 protein heterodimer, purified from the recombinant baculovirus-infected Sf9 cells, showed the same high degree of processivity of deoxynucleotide polymerization as the enzyme purified from the HSV-1 infected primate cells. Like the latter, it contained a 3′-5′ exonuclease activity that specifically hydrolyzes an incorrectly matched nucleotide at the 3′ terminus of a primer, thereby contributing to the fidelity of DNA replication.

Keywords: Exonuclease, Fidelity, HSV-1 Pol-UL42, Processivity.

Introduction

The herpes simplex virus type-1 (HSV-1) genome is a linear 153 kb DNA duplex. Of the approximately 75 open reading frames, the products of seven have been shown to be necessary and sufficient for replication of the viral genome. These gene products are a single-strand DNA-binding protein (ICP8), a heterotrimeric helicase-primase (Crute *et al.*, 1988; Crute and Lehman, 1989), an origin-binding protein (Elias *et al.*, 1986; Olivo *et al.*, 1988), a DNA polymerase, and a duplex DNA binding protein (UL42 protein) (Elias *et al.*, 1986; Marsden *et al.*, 1987; Gallo *et al.*, 1988; Parris *et al.*, 1988). The 65 kDa UL42 protein exists in a 1:1 complex with the 136 kDa UL30 DNA polymerase polypeptide (Park *et al.*, 1982; Crute and Lehman, 1989; Gottlieb *et al.*, 1990; Hernandez and Lehman, 1990). The UL42 protein interacts with origin-binding protein (UL9 protein) (Monahan *et al.*, 1998) and the DNA polymerase subunit interacts with UL8 protein of the heterotrimeric helicase-primase (Mardsen *et al*, 1997). In addition to DNA polymerase activity, the DNA polymerase-UL42 protein complex has a proofreading activity that can function as a 3′-5′ exonuclease. The mutations in the Exo III motif of the DNA polymerase incorporated into the viral genome produced increased mutation frequencies (Hwang *et al.*, 1997).

We have coexpressed the genes for the DNA polymerase and UL42 protein using the baculorvirus-Sf9 insect cell system and purified the heterodimeric enzyme to near homogeneity in milligram quantities. The baculovirus-expressed enzyme complex is identical in its processivity and proofreading capacity to that of the complex isolated from HSV-1 infected mammalian cells.

Materials and Methods

Enzymes and DNA Oligonucleotides were purchased from Oligos Etc. Activated calf thymus DNA was prepared by treatment of calf thymus DNA (Sigma, St. Louis, USA) with DNase as previously described (Uyemura and Lehman, 1976) to generate DNA fragments approximately 200 bp in length. Heparin-Sepharose was prepared as described (Davison *et al.*, 1979). Restriction enzymes were purchased from New England Biolabs (Beverly, USA). Polynucleotide kinase, exonuclease-free T7 DNA polymerase, and Sequenase version 2.0 were obtained from Amersham Life Sciences (Arlington Heights, USA).

Buffers Buffer A contained 40 mM Hepes, pH 7.5, 2 mM

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EDTA, 2 mM EGTA, 1 mM DTT, 10 mM mercaptoethanol, 10 mM sodium bisulfite, pH 7.5, 10% glycerol, 2 μg/ml leupeptin and pepstatin A, 1 mM PMSF, and 1 mM aminobenzamidine·2HCl. Buffer B contained 40 mM Hepes, pH 7.5, 2 mM EDTA and EGTA, 5 mM sodium bisulfite, 1 μg/ml leupeptin and pepstatin A, and 10% glycerol.

Cells *Spodopotera frugiperda* cells (Sf9 cells, a generous gift from M. Summers, Texas A & M University) were maintained at 27°C in Grace's medium (GIBCO, Rockville, USA) supplemented with 0.33% TC yestolate (Difco, Rockville, USA), 0.33% lactalbumin hydrosylate (Difco), and 10% heat inactivated fetal bovine serum (GIBCO).

Purification and quantitation of oligonucleotides The 30 mer and 50 mer oligonucleotides were purified by electrophoresis through a 15% polyacrylamide gel, electroeluted from the gel with the Elutrap (Schleicher & Schuell, Keene, USA), concentrated with a Speedvac, and desalted by ethanol precipitation or by Bio-Spin 6 (Bio-Rad, Hercules, USA) gel filtration. The 30 mer was 5′-^{32}P labeled with polynucleotide kinase and [γ-^{32}P] ATP, and annealed to the 50 mer. The measured specific activity (cpm/pmol) was then used to calculate the concentration of the 30/50 mer. Based on the concentration and the A_{260}, the extinction coefficient was calculated to be 1.9×10^6. The same extinction coefficient was used for 30/50 mer containing a terminal deoxyadenylate (29A/50 mer). The concentration of the 30/50 mer was also determined by measuring labeled deoxynucleotide incorporation in the presence of an excess of HSV-1 DNA polymerase. The reaction mixture (100 μl) incubated at 37°C contained 40 mM Hepes, pH 7.5, 150 mM KCl, 12 mM $MgCl_2$, 0.1 μM 30/50 mer (based on the A_{260}), 2 μM [α-^{32}P] dATP, 0.5 μCi/μM, and 50–100 nM HSV-1 DNA polymerase. Aliquots (10 μl) were taken at 1 min intervals. The reaction was stopped by the addition of 10 μl of 0.5 M EDTA, pH 8.0, and [^{32}P] dAMP incorporation was measured as described below. Both methods agreed within 10%. The oligonucleotides used are shown in Scheme I.

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30/50mer    GCCTCGCAGCCGTCCAACCAACTCTACCCT
            CGGAGCGTCGGCAGGTTGGTTGAGATGGGATGAGTTTGAAGTAGGTACAC

29A/50mer   GCCTCGCAGCCGTCCAACCAACTCTACCC(A)
            CGGAGCGTCGGCAGGTTGGTTGAGATGGGATGAGTTTGAAGTAGGTACAC
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Construction of a recombinant baculovirus containing the HSV-1 DNA polymerase (UL30) gene Plasmid pTH1 containing the UL30 gene (Hernandez and Lehman, 1990) was treated with *Nde*I and the 3′ recessed end produced was filled in with T7 DNA polymerase (Sequenase). The resulting blunt ended linear DNA was digested with *Xba*I. A fragment of the appropriate size was isolated and ligated into the pVL1393 (Invitrogen, Carlsbad, USA) that had been treated with *Sma*I and *Xba*I. The resulting recombinant plasmid, pBK22, was used to generate the recombinant baculovirus AcMNPV/UL30, BKV22, according to the instructions provided by the manufacturer (Invitrogen). Confirmation that the UL30 gene was out of frame with the polyhedrin gene was obtained by dideoxy sequencing using the oligonucleotide 5′-ATGATAACCATCTCGCAA-3′, which corresponds to a sequence 74 bases upstream of the start codon of the polyhedrin gene as a primer.

The recombinant baculovirus containing the UL42 gene (AcMNPV/UL42) was kindly provided by Dr. Mark Challberg (NIH).

Overexpression and purification of the DNA polymerase-UL42 protein complex Sf9 cells were seeded into thirty 225-cm^2 flasks and allowed to grow to 60–70% confluence. After removal of the medium, each flask was inoculated with 30 ml of recombinant baculovirus, 15 ml of AcMNPV/UL30, and 15 ml of AcMNPV/UL42, each at 10^8 pfu/ml. After 3 h of incubation, the inoculum was replaced with 35 ml of the fresh medium. The infected cells were incubated for 72 h at 27°C, dislodged by shaking and collected by centrifugation at $1500 \times g$ for 10 min. The cell pellet (10 ml) was resuspended and homogenized with a Dounce homogenizer in 1.5 M NaCl in buffer A. The resulting cell extract was centrifuged for 40 min at 40,000 rpm using the Ti45 rotor. The supernatant was dialyzed against buffer A containing 0.1 M NaCl. The purification procedure used was that described by Crute and Lehman (1989) except that Q-Sepharose Fast Flow (Pharmacia, Uppsala, Sweden) was substituted for the gel filtration step. The HSV-1 DNA polymerase lacking the UL42 protein was purified using the same procedure as that used for the DNA polymerase-UL42 protein complex.

DNA polymerase assay DNA polymerase activity was measured in a reaction mixture (20 μl) that contained 40 mM Hepes, pH 7.5, 150 mM KCl, 12 mM $MgCl_2$, 0.2 mg/ml activated calf thymus DNA, 12.5 mM each of dATP, dCTP, dGTP, and [^{3}H] dTTP, 460–480 cpm/pmol. The reaction was started by the addition of 1.0 μl of fractions from column chromatography. Incubation was for 10 min at 37°C. One-half of the reaction mixture (10 μl) was directly spotted onto a DE 81 filter. The filters were washed three times with 0.5 M sodium phosphate, pH 7.5, and then with ethanol before drying. The labeled DNA product was measured by scintillation counting.

Measurement of processivity Singly-primed M13mp18 DNA was prepared by annealing a 5′-^{32}P labeled primer (5′-AAATACCGAACGAAC-3′) to single-stranded M13mp18 DNA. The reaction was then initiated by mixing 5 μl of solution A containing 5 nM enzyme, 1 nM DNA in 20 mM Hepes, pH 7.5, 40 mM KCl, and 5% glycerol with 5 μl of solution B containing 3.4 mg/ml activated calf thymus DNA, 4 mM $MgCl_2$, 40 μM each of dATP, dCTP, dGTP, and dTTP in 20 mM Hepes, pH 7.5, 40 mM KCl, and 5% glycerol. In the controlled reaction, activated calf thymus DNA (3.4 mg/ml) was added to solution A in place of solution B. The reaction was stopped by addition of 2 μl of alkaline gel loading buffer. The products were analyzed by 1% agarose gel electrophoresis under alkaline conditions. Following electrophoresis, the gel was dried and autoradiographed.

Single nucleotide incorporation and exonuclease assay The single nucleotide incorporation reaction was initiated by mixing 37 μl of solution A containing 40 nM HSV-1 DNA polymerase, 1 nM 5′-^{32}P labeled DNA (30/50 mer) in 40 mM Hepes, pH 7.5, 150 mM KCl, 2.5% glycerol with 37 μl of solution B containing 100 μM dATP and 12 mM MgCl2 in 40 mM Hepes, pH 7.5, 150 mM KCl, 2.5% glycerol. The reaction was stopped with 60 μl of 0.5 M EDTA, pH 8.0, using a rapid quench flow apparatus (KinTek Instruments, College Park, USA). For the exonuclease